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Review article

Roles of the conjunctiva in ocular drug delivery: a review of conjunctival transport mechanisms and their regulation☆

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Abstract

Conjunctiva plays many roles including protection of ocular surface, production of tear film, and a conduit for drug clearance (depending on drug properties) into the systemic circulation or for drug transport to the deep tissues of the eye. The conjunctiva, which is a moderately tight epithelium, endowed with various transport processes for the homeostasis of ions, solutes, and water in the conjunctival surface and tear film. Modulation of ion transport in the conjunctiva leads to alterations in transconjunctival fluid flow that may become useful for treatment of dry-eye state in the eye. As a possible drug delivery route to the posterior portion of the eye, conjunctiva is an attractive route due to both larger surface area than that of cornea and expression of several key transport processes. Tear contains D-glucose and many amino acids, in addition to the usual ions in the body fluids. Several ion-coupled solute transport processes for absorption of amino acids, D-glucose, monocarboxylate, nucleosides, and dipeptides are expressed in the conjunctiva. Thanks to the rich endowment of these transport processes, drug transport across the conjunctiva into the intraocular tissues may become quite feasible. Subconjunctival injection of microparticles and matrix materials (which allows sustained release of drugs) is shown to maintain reasonable levels of various drugs in the vitreous, perhaps attesting to the fact that conjunctiva per se may contribute as a part of multiple transport barrier(s) in ocular drug delivery. In addition, several conjunctival approaches have been investigated to optimize treatment of dry-eye syndrome and intraocular diseases, and more can be accomplished in the coming years.

Keywords: Conjunctival drug delivery; Carrier-mediated transport; Ion transport; Solute transport; Endocytosis; Subconjunctival administration; Iontophoresis

1. Introduction

The conjunctiva is a thin transparent mucous epithelial barrier, lines the inside of the eyelids, and covers the anterior one-third of the eyeball. The respective portion of conjunctiva is referred to as the palpebral and bulbar conjunctiva. The joining area between palpebral and bulbar conjunctiva is referred to as the fornix (forniceal conjunctiva) (Fig. 1). The conjunctiva is composed of two layers: an outer epithelium and its underlying stroma (substantia propria). The epithelium is covered with microvilli and consists of stratified epithelial cells of 5–15 layers. The epithelial cells at the apical side connect with each other by tight junctions that play a role in permeability barrier. The stroma (containing structural and cellular elements, including nerves, lymphatics, and blood vessels) loosely attaches to the underlying sclera. The exposed surface of the eye includes conjunctiva and cornea and is covered with the tear film. The conjunctiva contributes to the formation of the tear film by way of secreting substantial electrolytes, fluid, and mucins [1].

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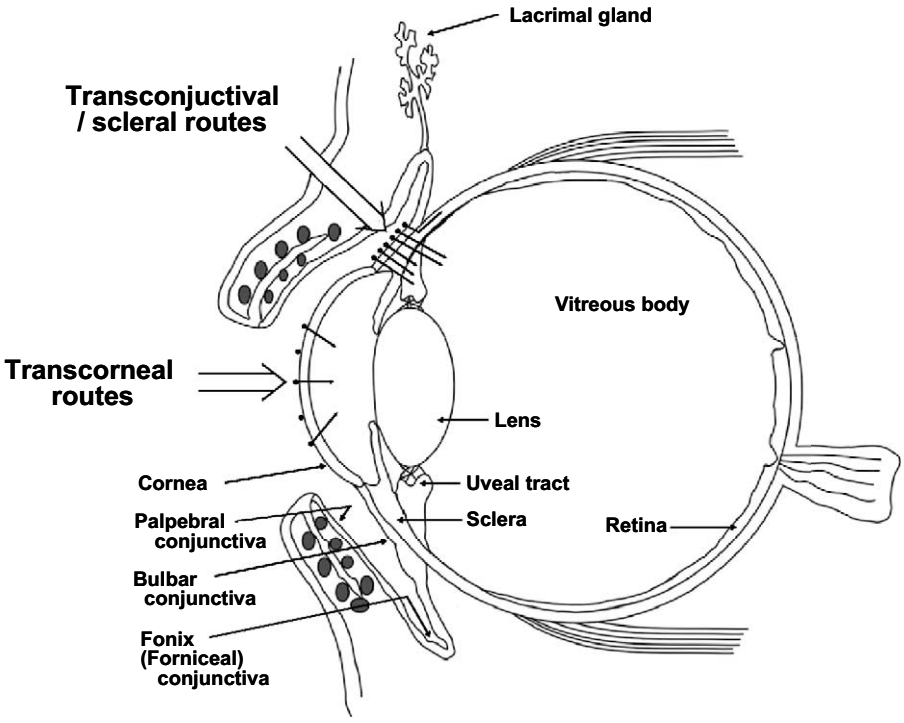

Fig. 1. Cross-section of eye and various drug absorption routes.

Topically applied drugs can reach the intraocular tissues by either the corneal and/or the non-corneal (conjunctival–scleral) pathways (Fig. 1). In animal studies, the ratio of the amount of lipophilic drugs in the iris–ciliary body absorbed through the respective corneal and non-corneal pathways is 70:1 for hydrocortisone [2], 12:1 for timolol [3], and 5:1 for pilocarpine [2]. By contrast, inulin, a hydrophilic model drug with a molecular weight of ~5000 Da is absorbed primarily by non-corneal pathways, as conjunctiva is much more permeable to hydrophilic solutes (e.g. D-mannitol and inulin) than the cornea [3,4]. Thus, the conjunctival–scleral pathway is favored for delivery of hydrophilic drugs, bypassing the anterior chamber and permitting direct access to the intraocular tissues of the posterior segments (e.g. uveal tract and vitreous humor) [5]. Interestingly, the surface area of the conjunctiva is ~9 and 17 times larger than that of cornea in rabbits and human, respectively [6], which may be another contributing factor for greater absorption of hydrophilic drugs via conjunctival routes, as conjunctiva is endowed with leakier and more numerous tight junctions than cornea. Since 1994, several transporters (e.g. PepT1, P-glycoprotein, and amino acid transporters) have been recognized that may play important roles in achieving influx and efflux transport of drugs (and other metabolites) in the conjunctiva.

Compared with corneal drug transport research, conjunctival research is relatively unexplored. This lack of attention in the vision research community is somewhat surprising, given that the conjunctiva is unavoidably involved whenever topical applications of many drug formulations are used to treat anterior segment diseases. Since 1993, we have been studying the transport properties of the conjunctiva in the context of drug absorption. With few exceptions, all the data were obtained in vitro using the conjunctivas excised from pigmented rabbits and primary cultures derived thereof. Consequently, there is a lot to be learned from humans in the coming years.

In this review, we shall focus on transport characteristics of the conjunctival epithelium, including passive barrier properties, evidence for active solute transport processes (including efflux pumps), active ion transport and its regulation/modulation, and fluid transport characteristics. The feasibility for intraocular drug delivery via conjunctival routes (utilizing carrier-mediated transport and passive diffusion process) is discussed. Finally, we included assessment on subconjunctival administration and transscleral/conjunctival iontophoresis, where we deem appropriate. We would like to point out the fact that these latter subject matters are not directly relevant for conjunctival drug transport, but may explain the formidable role of conjunctiva in ocular drug delivery.

2. Barrier properties of the conjunctiva

Paracellular transport of hydrophilic solutes (including ions and water) across the conjunctiva may be rather limited, since conjunctival epithelial cells are joined by tight

junctions at the apical-most aspect of the epithelium. Tight junctions form barriers between adjacent cells and act as a major impediment for diffusion of hydrophilic drugs via paracellular pathways [7]. Horibe et al. [8] studied transport properties of several hydrophilic model drugs (e.g. D-mannitol (182 Da), 6-carboxyfluorescein (376 Da), and fluorescein isothiocyanate-labeled dextrans (FD) with molecular weights of 4400 (FD4), 9400 (FD10), 21,300 (FD20), and 38,600 Da (FD40)) to find that transport rates of these solutes across the conjunctiva lack both concentration- and direction-dependency. Permeability coefficients (P_{app}) of these hydrophilic solutes decrease with increased solute sizes (i.e. molecular weights). Equivalent pore analysis, assuming restricted solute diffusion via cylindrical, water-filled pores across the freshly excised conjunctival tissues, reveals an equivalent pore radius of 5.5 nm with a density of 1.9×10^8 pores/cm^2. Hämäläinen et al. [9] also reported that pore radius for corneal epithelium, palpebral conjunctiva, and bulbar conjunctiva of 2.0, 4.9, and 3.0 nm, respectively. For hydrophilic drugs with molecular weights <550, molecular radius is less than 2.0 nm, whereas FD20 has a radius of 4.9 nm [8]. Therefore, the conjunctiva may allow the permeation of hydrophilic substances with a molecular weight less than 20,000 [4,8], whereas cornea is not permeable to inulin ($\sim$5 kDa) and FD20 [4].

Tissue resistance is a good indicator of passive barrier properties of a given biological barrier. Kompella et al. [10] reported that the freshly excised pigmented rabbit conjunctiva is a moderately tight epithelium with a transepithelial electrical resistance (TEER) of $\sim$1.3 kΩ cm^2. This value is in general agreement with the report by Shi and Candia for the excised albino rabbit conjunctival tissues [11]. The conjunctival tissues excised from pigmented rabbits have a lower TEER than the more tighter rabbit cornea whose TEER is $\sim$7.0–9.0 kΩ cm^2 [12,13]. The rabbit conjunctiva in general is more permeable to several hydrophilic solutes than cornea, as demonstrated earlier in vivo by Maurice [14].

3. Transport functions of conjunctiva

Although in vivo approach to study transport of drugs from ocular surface to intraocular tissues under physiological conditions, utilizing rabbits and rats by topical instillation to assess the absorption capacity of conjunctiva/cornea, has been reported by several laboratories [15–17], dissection of transport routes (e.g. corneal vs. the non-corneal (i.e. conjunctival–scleral) pathways) and their associated transport mechanisms from such data are almost impossible. In recent years, mechanistic studies of these potential transport routes have been accomplished by using both freshly excised tissues (of cornea and conjunctiva) and in vitro cultivation of respective epithelial cells on permeable substrata under primary culture conditions [10,18–20].

As compared to excised conjunctival tissues, primary cultured rabbit conjunctival epithelial cells (RCEC) allow one to study the epithelial barrier free of underlying stroma that contains non-epithelial elements of nerves, lymphatics, and blood vessels. Moreover, edge effects, which are inevitably present when mounting isolated tissues in Ussing-type chambers can be reduced or eliminated altogether with cells cultured in permeable filter cups, as no direct mechanical pressure is exerted on the cells themselves. An added bonus for RCEC may be the mass production of more or less homogeneous cell models in vitro that can be utilized simultaneously for transport studies out of one animal, lessening the variances associated with animal-to-animal differences in transport properties. RCEC exhibit apical presence of microvilli, and a small population of mucin-secreting cells interspersed with the majority of surface epithelial cells with stratified structure, akin to the in vivo situation. RCEC layers show a TEER of $\sim$1.9 kΩ cm^2, potential difference (PD) of $\sim$14 mV (apical negative), and an equivalent short-circuit current (I_{sc}) of $\sim$8.0 μA/cm^2 [18]. These bioelectric properties mimic well with those properties of freshly excised tissues, as the electric resistance and permeability of drugs with low molecular weight of RCEC layers are quite similar to those of excised tissues. RCEC grown on permeable support have been widely used to study transport mechanisms for several model drug compounds [20,21]. Several pieces of evidence support transport processes summarized in Fig. 2 for active transport of solutes and ions across conjunctival tissues.

3.1. Secondary active solute transport

3.1.1. D-Glucose

3-*O*-methyl-D-glucose (3-OMG, a non-metabolizable analog of D-glucose) transport across the pigmented rabbit conjunctiva in the mucosal-to-serosal (ms) direction appears to be a carrier-mediated process with a 1:1 stoichiometry between Na^+ and 3-OMG. Evidence for such co-transport process includes strong temperature-dependence, saturability with a K_m of 16.7 mM 3-OMG, directionality where ms flux of 3-OMG is greater than that in the opposite (i.e. sm) direction, dependence on mucosal presence of Na^+, and inhibition by serosally added 0.5 mM ouabain and mucosally added 0.5 mM phlorizin [22]. In addition to these functional evidences, Turner et al. provided morphological evidence by immunofluorescence microscopy, showing that Na^+-D-glucose transporter (SGLT1) is localized on the mucosal side of the conjunctiva [23]. Although Na^+-D-glucose co-transport has not been identified in any other ocular tissues to date, facilitative glucose transporter (GLUT1) has been reported in the corneal endothelium [24], lens [25], iris–ciliary body [26], retinal capillary and retinal pigmented epithelium [27]. The role of such glucose transporter(s) in transconjunctival fluid secretion/absorption is discussed elsewhere in this review.

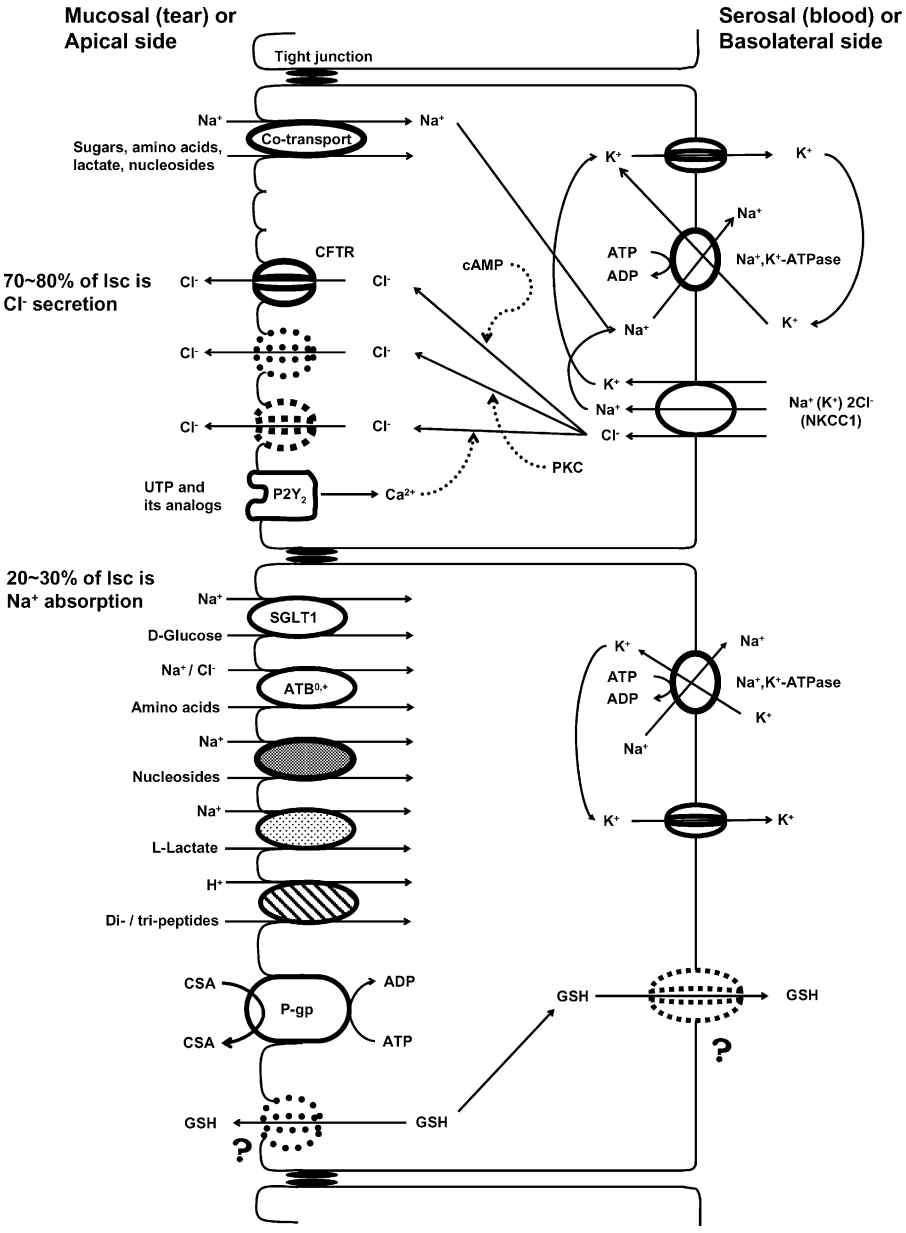

Fig. 2. Ion and solute transport processes in the conjunctiva. Some transport processes (e.g. Na^+-independent, carrier-mediated processes) are not shown for clarity, especially those localized at the serosal (or basolateral) aspect of the conjunctiva.

3.1.2. Amino acids

Liaw et al. [28] reported that Na^+-coupled L-lysine (L-Lys) transport across the rabbit corneal epithelium occurs via a carrier-mediated process with a 1:1 stoichiometry between Na^+ and L-Lys, with temperature-dependence, saturability, directionality, Na^+-dependence, and inhibition by serosally added ouabain. It is worth noting that Na^+-α-aminoisobutyric acid transport is found in the toad corneal epithelium [29], although conjunctival presence of such neutral amino acids has not been reported to date. As about 352 μM (in total) amino acids are known to be present in the human tear fluid [30], more amino acid transport processes may become unraveled in the future. Bioelectric studies of excised conjunctivas of pigmented rabbits (mounted in Ussing-type chambers) first suggested the possible existence of Na^+-amino acid co-transport process(es) in the mucosal aspect of conjunctiva [31]. Of the several amino acids tested, L-arginine (L-Arg) (which is known to be present at ∼18 μM in the human tear fluid [30]) elicited the largest I_{sc} increase in the conjunctiva in response to mucosal addition into the amino acid-free Ringer's solution [31]. Subsequently, Hosoya et al. [32] further investigated Na^+-coupled L-Arg transport mechanisms and found that L-Arg transport at 37 °C in the ms direction is comprised with both high- and

low-affinity processes with respective K_m's of 0.07 and 5.9 mM. Moreover, these investigators reported that saturability was not observed under mucosal Na^+-free condition and at 4 °C, while directionality, ouabain sensitivity, and inhibition of [^{3}H]L-Arg transport by excess unlabeled L-Arg added to the mucosal fluid, all supported the presence of carrier-mediated L-Arg transport in the mucosal cell membranes of conjunctiva of the pigmented rabbits. Based on Hill analysis of L-Arg transport, a 1:1 coupling between Na^+ and L-Arg was noted, and the pattern of inhibition by basic and large neutral amino acids along with Na^+-dependency suggests the presence of system $B^{0,+}$ ($ATB^{0,+}$)-mediated L-Arg transport in the pigmented rabbit conjunctiva. Morphological evidence, provided by Hatanaka et al. who determined immunolocalization of $ATB^{0,+}$ in the eye, indicates that abundant $ATB^{0,+}$ is localized on the apical side of conjunctiva (as well as in retinal ganglion cells, retinal pigmented epithelium, and inner nuclear layer of the retina) [33]. These amino acid transport processes are likely to play important roles in scavenging the amino acids normally present in the tear fluid. We currently do not know the role of such amino acid transporters in the case of non-physiologic and/or pathophysiological states of the surface of the ocular tissues, when excess amount of such solutes might be prevalent.

3.1.3. Nucleosides

It is well known that Na^+-dependent adenosine and guanosine co-transport process(es) are present in rat retinal cells [34], although neither the presence of such co-transport mechanisms in other ocular epithelia nor the nucleoside concentrations in tear fluid are known to date. Hosoya et al. [35] for the first time described the conjunctival presence of Na^+-dependent nucleoside transport system using the freshly excised tissues from pigmented rabbits. These investigators reported that uridine transport in the ms direction across conjunctiva exhibits strong directionality, temperature dependency, and phlorizin sensitivity. Interestingly, uridine transport appeared to be mediated via both Na^+-dependent and Na^+-independent (but nitrobenzylthioinosine-insensitive) processes with respective K_m's of 1.9 and 200 μM. More importantly, uridine increased the I_{sc} in a concentration-dependent manner with a K_m of 7.6 μM and maximal increase in I_{sc} of 0.7 μA/cm^2. Based on a 1:1 stoichiometry and of the stronger inhibition of [^{3}H]uridine transport by purine than pyrimidine nucleotides, these investigators suggested that CNT2 (cif, N1) type of Na^+-dependent nucleoside transport process [36] probably predominates in the pigmented rabbit conjunctivas. The role of such nucleoside transport processes may become important in accurately assessing efficacies of exogenous nucleoside drugs in the eye in general.

3.1.4. L-Lactate

The concentration of lactate in tear fluids is about four to ten times higher than that in serum, while pyruvate levels are about the same in the two fluids [37]. Horibe et al. [38] reported that L-lactate transport in the ms direction across the pigmented rabbit conjunctiva most likely takes place via a carrier-mediated process (e.g. Na^+-dependent monocarboxylate transport) localized in the mucosal aspect of conjunctivas. Evidence for such lactate transport process includes strong directionality, temperature-dependence, Na^+-dependence, and saturability with a K_m of 1.9 mM lactate. L-Lactate may be transported across the conjunctiva with $2Na^+$ and transconjunctival epithelial lactate transport is inhibited by acetate, pyruvate, propionate, benzoate and nicotinate. Na^+-dependent monocarboxylate transport process is known to be present in rabbit corneal endothelium [39], rabbit kidney [40], and rabbit small intestinal brush border membrane [41]. H^+-L-lactate co-transport process has been recognized in the corneal epithelium and endothelium, which may play a role in removal of lactate from highly glycolytic cornea and may participate in modulation of intracellular pH [39,42]. Monocarboxylate transporter (MCT) 1 and 3 have been found from retinal capillary and retinal pigmented epithelium, suggesting a role for modulating lactate levels in the interphotoreceptor space [43,44]. The exact nature of conjunctival monocarboxylate transport process(es) remains unknown to date.

3.1.5. Peptide transport

Oligopeptide transporters (e.g. PepT1 and PepT2) are H^+-coupled co-transport systems and play a role in absorbing di- and tri-peptides, and dipeptide-mimetic drugs in the intestinal [45] and renal [46] epithelial cells. Sun et al. reported that dipeptide transport process in the pigmented rabbit conjunctiva transports L-carnosine (β-alanyl-L-histidine) across the excised pigmented rabbit conjunctiva by a carrier-mediated and H^+-dependent process, resulting in an increase of I_{sc} upon mucosal instillation of the dipeptide [47]. Using RCEC, temperature-, H^+-, and concentration-dependent process(es) with a K_m of 0.3 mM dipeptide was described [48]. Dipeptide transport process is inhibited by a proton ionophore, a number of other dipeptides and dipeptide-mimetic drugs, suggesting that H^+-coupled dipeptide transport process(es) may indeed be present in the pigmented rabbit conjunctiva. Mechanistic details of dipeptide transport in ocular tissues remain largely unknown.

Although glutathione (GSH) is a tripeptide composed of L-glutamic acid, L-cysteine, and glycine, it is not a substrate for dipeptide transporters due to the peculiar structure of this tripeptide, in which the joining of L-cysteine residue and γ-carboxyl group of L-glutamic acid is unique. In many epithelial (and endothelial) barriers, metabolism and transport of GSH, the endogenous thiol antioxidant, are now well characterized, in that GSH plays important roles in antioxidant defense as well as cellular homeostasis. Very recently, our laboratories reported conjunctival GSH secretion [49] and transport properties of one of its constituent amino acids, L-cyst(e)ine [50], using primary

RCEC model. Apical uptake and efflux of radiolabeled GSH turned out to be mostly (up to 65%) Na^+-dependent. GSH uptake rate was higher from apical fluid than from basolateral fluid. A coupling ratio for Na^+:GSH of 1.25:1 was estimated by Hill analysis. Interestingly, however, GSH efflux into the apical fluid was marginally dependent on apical presence of Na^+ and significantly greater than that into basolateral fluid. Basolateral efflux of GSH was primarily Na^+-independent, whereas basolateral uptake almost exclusively was Na^+-dependent. Moreover, depolarization of the cell membrane potential decreased GSH efflux into either apical or basolateral fluids (5 pmol/min per 10^6 cells), while hyperpolarization significantly increased the rate of GSH efflux into the apical fluid (120 pmol/min per 10^6 cells) without any effect on the basolateral efflux. These polarized efflux/uptake mechanisms for GSH transport in conjunctival epithelial cells dictate the overall GSH transport across the epithelial barrier, in that apparent permeability of GSH across RCEC layers was ~8 times higher in the basolateral-to-apical (secretion) than the opposite (absorption) direction. These data strongly suggest that GSH is transported across RCEC membranes by both Na^+-dependent (with 1:1 coupling) and Na^+-independent processes. Interestingly, the Na^+-independent component is highly sensitive to cell membrane potential. We currently do not have any information on such transporter(s) that are responsible for moving GSH into or out of cells, although a couple of reports that implicate cystic fibrosis transmembrane conductance regulator (CFTR) as one of the GSH conducting mechanisms in some cells [49,51]. As hyperpolarization of cell membrane alters GSH efflux, it is attractive to postulate that high conductance anion channels that are responsible for ATP efflux may also allow GSH efflux from cells. We further suggest that net secretion of GSH into the apical fluid is expected to help protect conjunctival tissue as well as tear film from extraneous oxidant insults.

For the metabolism and maintenance of intracellular GSH at an appropriate level, the transport into the cells of one of the constituent amino acids, cyst(e)ine, becomes critical in health and disease of conjunctiva. We recently reported that L-cystine is transported by both Na^+-dependent and -independent amino acid transport systems in RCEC [50]. At low substrate concentrations, L-cystine uptake was higher from apical than basolateral fluid. Permeability studies indicate that L-cystine is net absorbed across RCEC. S-nitroso-*N*-acetylpenicillamine (SNAP, NO donor) caused significant increases in both L-cystine uptake and intracellular GSH level, which occurred concomitantly with elevation of both catalytic and regulatory subunits of γ-glutamylcysteine synthetase (GCS). Understanding sulfur amino acid precursor-dependent cellular mechanisms of GSH homeostasis would be of value in devising GSH-based treatment for conjunctival or other ocular disorders [50].

Some basic mechanisms of H_2O_2-induced reduction in rates of active ion transport (I_{sc}) across the pigmented rabbit conjunctival tissue and the protective role afforded by mucosal GSH have been also reported. A higher mucosal half inhibition concentration (IC_{50}) for H_2O_2 on conjunctival I_{sc} corresponds to the faster consumption of exogenous H_2O_2 from mucosal bathing fluid. In addition, actively secreted GSH by conjunctival epithelial cells may help reduce the injury by mucosally applied H_2O_2. Serosal injury by H_2O_2 may directly and irreversibly affect vital membrane components (e.g. Na^+,K^+-ATPase) involved in active ion transport across conjunctiva. Mucosal protection by GSH (or its analogs) of active conjunctival ion transport may be useful in maintaining physiological functions of conjunctiva under oxidative stress [52], although the exact mechanisms by which cellular GSH homeostasis is maintained is largely unknown to date.

3.2. Efflux transporter (e.g. P-glycoprotein)

P-glycoprotein (P-gp) is an ATP-dependent 170 kDa membrane glycoprotein associated with multidrug resistance in (mostly) tumor cells, which is responsible for decreasing the intracellular accumulation of a wide variety of chemotherapeutic agents and hydrophobic compounds [53]. P-gp is also expressed in normal tissues where it possibly serves a protective role against the entry of foreign (i.e. xenobiotic) compounds. Transport rates of cyclosporine A (CSA) and propranolol observed in the basolateral-to-apical direction of RCEC were reported to be greater than those in the opposite direction [54,55], suggesting that secretory mechanisms may be in operation. Net secretory flux of these drugs appears to be due to an energy-dependent mechanism, since P-gp inhibitors and substrates (e.g. verapamil, progesterone, CSA, and anti-P-gp monoclonal antibody (4E3 mAb)) all decrease the net secretory flux of the model drugs, when added to the apical fluid. Moreover, net secretory flux of propranolol is saturable with a K_m of 72 nM and immunofluorescence studies reveal positive staining in the apical cell membrane of RCEC (and in the mucosal surface of the superficial cell layers in the excised pigmented rabbit conjunctiva) [55]. P-gp-mediated drug efflux pump on the apical cell plasma membrane of the conjunctiva plays a role in restricting the conjunctival absorption of some lipophilic drugs and xenobiotics. Modulation of such efflux mechanisms in conjunction with treatment of ocular tissues in diseases remain a big challenge in the coming years.

3.3. Ion transport

3.3.1. Cl^- transport

Kompella et al. [10], using Ussing-type chamber and voltage clamp techniques, demonstrated a spontaneous PD of 17.7 ± 0.8 mV (tear-side negative), I_{sc} of 14.5 ± 0.7 μA/cm^2, and TEER of 1.3 ± 0.1 kΩ cm^2 in the freshly excised conjunctivas of pigmented rabbits. These values are in general good agreement with the report by Shi and Candia

[11] on excised conjunctival tissues of the albino rabbit. The PD data obtained in excised conjunctivas are reflective of the transepithelial potential values measured in vivo in the albino rabbit conjunctiva, ranging from 0 to 25 mV (tear side negative) with a mean value of 15 mV [14]. The pigmented rabbit conjunctiva was capable of actively secreting Cl^-, accounting for 70–80% of the conjunctival short-circuit current (I_{sc}, which is an index of active ion transport rates), as evident from ~70–80% inhibition of I_{sc} in either serosal absence of Cl^- or mucosal presence of 4 mM *N*-phenylanthranilic acid (NPAA, an inhibitor of epithelial Cl^- conductive pathways) [10]. About 20–30% of conjunctival I_{sc} is contributed by Na^+ absorption via Na^+-coupled co-transport process(es) (e.g. Na^+-D-glucose co-transport, as indicated by 20–30% reduction in I_{sc} by absence of either Na^+ or D-glucose from the mucosal Ringer's solution (containing 141 mM Na^+ and 5 mM D-glucose, but no amino acids)) (Fig. 2) [56].

As for active Cl^- secretion across the conjunctiva, Cl^- enters epithelial cells via serosal $Na^+(K^+)2Cl^-$ co-transport process. This is based on the observation that conjunctival I_{sc} is inhibited in a concentration-dependent manner by serosally instilled bumetanide with an IC_{50} of ~7 μM and maximal I_{sc} inhibition of ~60% [10]. In support of this functional evidence, $Na^+(K^+)2Cl^-$ (NKCC1) co-transport process was shown to be present in the serosal aspect of rabbit conjunctiva by immunofluorescence [23]. The presence of K^+ channel activity in the serosal aspect of the conjunctiva is supported by the inhibitory effect of serosally administered Ba^{2+} on I_{sc} with an IC_{50} of 2 mM, suppressing K^+ recycling between the cell interior and the serosal fluid [10].

Mucosal exit of Cl^- from conjunctival epithelial cells occurs via Cl^- channels, as mucosally added NPAA inhibits I_{sc} in a concentration-dependent manner with an IC_{50} of 0.3 mM and maximal inhibition at 80% [10]. Such pharmacological evidence for Cl^- transport was further confirmed by unidirectional ^{36}Cl flux studies, where net Cl^- secretion across the conjunctiva is abolished by mucosal application of 1 mM NPAA and serosal application of 10 μM bumetanide [57]. One of the Cl^- channels, CFTR, has been confirmed to be expressed in the mucosal aspect of rabbit conjunctivas by immunofluorescence [58,59].

3.3.2. Regulation/modulation of active Cl^- secretion across conjunctiva

Active secretion of conjunctival Cl^- secretion is modulated by various agents. Mucosally added 1 mM 8-BrcAMP increases I_{sc} by 143% [60]. A role for cAMP modulation of conjunctival I_{sc} is further supported by the 81 and 33% increase in I_{sc} by mucosal presence of 2 mM theophylline and 2 μM epinephrine, respectively, and the 86% increase in I_{sc} by 15 μM forskolin [60]. Net Cl^- secretion estimated under open-circuited conjunctivas at baseline by ^{36}Cl flux studies is ~0.15 μEq/cm^2 per h, which is stimulated by 133% with 1 mM 8-BrcAMP, 107% with 10 μM A23187 (Ca^{2+} ionophore), and 87% with 1 μM phorbol 12-myristate-13-acetate (PMA, PKC activator), respectively [57]. These data suggest that cAMP, Ca^{2+}, and PKC all modulate active Cl^- secretion, albeit the detailed signaling mechanisms for such stimulation largely remain unknown.

Active Cl^- secretion across conjunctivas is also modulated by instillation of mucosal (but not serosal) application of exogenous nucleotides. For example, mucosal presence of uridine 5′-triphosphate (UTP) also increases I_{sc} in a concentration-dependent manner with a half-maximal concentration (EC_{50}) of 11 μM and maximal increased I_{sc} of 107%, whereas UTP had no effects from the serosal side [61]. In addition, net Cl^- secretion estimated under short-circuited ^{36}Cl transport was 0.17 μEq/cm^2/hr and 100% increased from baseline in the mucosal presence of 10 μM UTP [61]. UTP, acting through $P2Y_2$ and/or $P2Y_4$ receptors and phospholipase C-sensitive Ca^{2+} signaling pathways, but not cAMP-sensitive pathway, stimulates net Cl^- secretion in the conjunctiva. $P2Y_2$ receptor mRNA, which is determined by in situ hybridization, is localized in the rabbit conjunctiva [62]. $P2Y_2$ receptor agonists, such as UTP and UTP analogs, stimulate Cl^- and fluid secretions in the conjunctiva [61,63], lending credence to a possibility that nucleotides may be used therapeutically to augment cAMP-independent Cl^- and fluid secretion in the conjunctiva of dry eye (syndrome) as ATP or UTP activates Cl^- secretion in normal as well as cystic fibrosis airway epithelia [64]. Cystic fibrosis is characterized by abnormalities of ion and fluid transport due to mutations in the gene coding for the CFTR protein, which functions as a cAMP-regulated Cl^- channel [65]. These data clearly suggest that active chloride secretion into tear side of the conjunctiva may be modulatable by a number of factors including cAMP, Ca^{2+}, PKC, and exogenous nucleotides (via $P2Y_2$ type purinergic receptor).

3.3.3. Na^+ transport

Na^+-coupled D-glucose co-transport contributes Na^+ absorption from the tear side of the conjunctiva, as reduction of I_{sc} is noted in the mucosal absence of either Na^+ or D-glucose [11,56]. As human tear fluid is known to contain about 0.4 mM D-glucose under normal conditions [66], D-glucose at 0.4 mM may contribute ~0.8 μA/cm^2 to the I_{sc} across the rabbit pigmented conjunctiva [56]. It may be worthwhile to note that amiloride-sensitive Na^+ entry into cells via Na^+ channels in the mucosal aspect of conjunctival epithelium does not appear to be in operation, as mucosally applied 1 mM amiloride (a Na^+ channel blocker) does show any effect on I_{sc} [10]. Other types of non-specific cation channels may be expressed in conjunctiva, although clear evidence is still lacking to date.

Na^+ absorption at baseline (i.e. in the presence of 5 mM D-glucose) has been estimated under short-circuited conditions by studying ^{22}Na fluxes [22]. At baseline, Na^+ transport in the ms direction is significantly greater than that

in the opposite direction, indicating net absorption of Na^+ across conjunctivas. Net absorption of Na^+ of ~0.15 μEq/cm^2/hr is consistent with the fraction of I_{sc} observed of 4.0 μA/cm^2 (the difference in I_{sc} in the presence and absence of Na^+ in mucosal bathing fluid). Moreover, net Na^+ absorption is abolished by serosally added 0.5 mM ouabain (a Na^+,K^+-ATPase inhibitor), whereas mucosally added 0.1 mM amiloride did not affect Na^+ fluxes in either direction. Mucosal presence of 0.5 mM phlorizin (and D-glucose) or mucosal absence of D-glucose significantly inhibited Na^+ flux in the ms direction by 40–60%. These data corroborate with the bioelectric studies [56]. Moreover, Na^+-amino acid co-transport in the conjunctiva also contributes net Na^+ absorption from the tear fluid, as L-Arg at 1 mM stimulates net Na^+ absorption by 0.12 μEq/cm^2 per h (or an increase in I_{sc} of ~3.0 μA/cm^2) [32].

3.4. Fluid transport

Net active Cl^- secretion, which is the predominant ion transport function of the conjunctiva [10,11,57], appears to be the definitive driving force for net fluid secretion into tear fluid across the conjunctivas. Fischbarg [67] hypothesized that corneal transendothelial fluid transport is pulsatile and regulable by cyclic, sequential transient activation of two different sets of osmolyte (ion and solute) transporters and/or that water channels may be involved (e.g. triggered by changes in cell volumes). $HgCl_2$-sensitive water-channel is expressed in the frog cornea [68] that is further characterized by cloning and subsequent expression in *Xenopus laevis* oocytes [69]. AQP 1 (water channel type 1 or channel-forming integral membrane protein of 28 kDa, CHIP 28) is also found in the corneal endothelium, lens epithelium, non-pigmented epithelium of the ciliary process, and iris epithelium [70]. AQP 3 (or glycerol intrinsic protein, GLIP) is reported to be present in the serosal (but not mucosal) side of rat conjunctiva and confirmed by immunohistochemistry [71]. Fluid transport in the conjunctiva is unclear, as to which type(s) of water channels are involved and/or what kinds of osmolyte(s) are important in causing such fluid movement. It is not quite understood as to how a number of signaling-related agents or molecules regulate/modulate fluid transport. A recent review (and references cited therein) on ion transport and fluid movement may be consulted for further reading [72].

The rate of fluid flow across a freshly excised pigmented rabbit conjunctiva, measured by a pair of capacitance probes in a modified Ussing-type chamber, in the sm direction under baseline conditions is ~4.3 μl/h per cm^2 [73]. Either 1 mM 8-BrcAMP or 10 μM UTP, when applied mucosally, stimulates conjunctival fluid secretion by ~2-fold. By contrast, either serosally applied 0.5 mM ouabain or serosal Cl^--free conditions rendered the fluid secretion to cease. When active Na^+ absorption is stimulated with mucosally applied 20 mM D-glucose, net fluid secretion rate decreased down to ~1.0 μl/h per cm^2. By contrast, 20 mM D-mannitol did not affect fluid transport across conjunctival epithelium. These data are consistent with a thesis that pigmented rabbit conjunctiva secretes fluid, secondary to active Cl^- secretion. As this net fluid secretion is subject to modulation by changes in either active Cl^- secretion or mucosal fluid composition (e.g. D-glucose concentration), the drugs to be applied topically to conjunctival (or corneal) regions should be formulated with a caution, i.e. agents potentially interfering with active secretion of Cl^- or stimulating absorption of Na^+ should be avoided, which may cause decrements in conjunctival fluid secretion as a result [73].

It is important to stress the fact that cAMP-dependent Cl^- transport and other Na^+-coupled solute (i.e. D-glucose and amino acids) transport may play a key role for maintenance of normal conjunctival cell volumes as well as net fluid balance across the conjunctiva. In particular, cAMP-independent Cl^- secretion (via purinoceptor activation) may be utilized to stimulate net fluid secretion (and thus augment tear fluids) across the conjunctival epithelial barrier. Moreover, nucleotides (i.e. UTP and UTP analogs) may become therapeutically useful agents to induce alternative net Cl^- and fluid secretion in several diseases (e.g. dry eye and/or cystic fibrosis) [74]. Clearly more mechanistic studies are required to make these processes to be of therapeutic use in the near future.

4. The role of conjunctiva in drug delivery to the posterior segment of the eye

4.1. Topical application of drugs to conjunctiva/cornea

Topically applied drugs can reach the intraocular tissues by either the corneal and/or the non-corneal (conjunctiva–scleral) pathways (Fig. 1). However, it is common to see about 1% or less of an applied dose absorbed across the cornea and conjunctiva to reach the anterior segment of the eye [75]. Obviously, subsequent movement of absorbed drugs to the posterior segment of the eye may occur for only a tiny fraction of that in the anterior segment. In addition, an instilled dose is also eliminated from the pre-corneal area within ~90 s and absorbed systemically by way of the highly vascular conjunctival stroma. Systemic absorption also occurs in the nasal mucosa when the solution drains the nasolacrimal duct [15,76,77]. Although several hurdles exist for intraocular drug delivery by the conjunctival pathway, we challenged to investigate for delivering drugs to the posterior segment of the eye. We found that nipradilol, a β-blocker, and iganidipine, Ca^{2+} antagonist are good examples to reach the posterior segment of the monkey and rabbit eye, respectively, although the mechanism of transport is not known yet, when both are topically applied [78,79]. We summarized below the mechanisms of drug transport in the conjunctiva to overcome the elaborate defense systems.

4.1.1. Passive transport of drugs via conjunctiva

Improving the conjunctival drug permeability is one of the major challenges in ocular drug delivery. In the past, the effort has been focused on either enhancing transcellular drug penetration by increasing drug lipophilicity through the use of prodrugs [80] or analogs [81] or improving paracellular penetration by using enhancers to open tight junctions [82]. Apparently, lipophilic drugs are absorbed far better than hydrophilic drugs via the transcellular route. A lipophilic drug, propranolol with a log partition coefficient between octanol and water (log PC) of 3.21, is absorbed through cornea and conjunctiva 5–10-fold greater than a hydrophilic drug, sotalol (whose molecular weight is about the same as propranolol) with a log PC of −0.62. The penetration of β-blockers through the conjunctiva and cornea increases with lipophilicity following a sigmoidal relationship [81]. Since hydrophilic drug penetrates only via paracellular pathway, i.e. between the cells through the tight junctions, the penetration area for any hydrophilic drugs is extremely small compared to the surface area offered by transcellular routes for absorption of lipophilic drugs [83,84]. However, some lipophilic drugs undergo efflux afforded by P-gp localized on the apical side of conjunctiva [54,55], necessitating the determination of a given lipophilic drug if it is a substrate for P-gp.

Peptide and protein drug delivery via ocular routes has been studied by pharmaceutical investigators as an alternate administration route to parenteral route [85]. Peptide and protein drugs are thought to be transported across ocular routes in part via paracellular pathways due to their hydrophilic nature [86,87]. Paracellular pathways of epithelial barriers like the cornea and conjunctiva are where neighboring cells are held together at the apical aspect by tight junctions [4], which act as the rate-limiting step for diffusion of hydrophilic macromolecule drugs including several peptides and proteins [7]. Equivalent pore analysis suggests that conjunctiva may allow the permeation of hydrophilic substances of a molecular weight $<$20,000 Da (whose molecular radius is $\sim$4.9 nm), as the equivalent pore radius is about 5.5 nm [8]. The penetration of peptide drugs across the conjunctiva is restricted by not only the structural barrier but also an external enzymatic barrier [88]. For example, ocularly applied enkephalins are significantly degraded by enzymatic activity [89]. Co-application of protease inhibitor(s) may be one way to increase absorption of peptide and protein drugs, as the presence of camostat mesylate (an aminopeptidase inhibitor) and leupeptin (a serine protease inhibitor) in the mucosal fluid yield absorption of intact arginine vasopressin transport across the pigmented rabbit conjunctiva [90]. Lastly, it is worth noting that other modes of protein absorption, i.e. via endocytosis routes, has not been well characterized to date, although recent reports indicate that ocular epithelial tissues may also be endowed with such vesicular routes for absorption of proteins. The roles and fractions of caveolae-mediated, clathrin-coated, and other vesicular transport routes in absorption/secretion of protein drugs remain largely unknown to date.

4.1.2. Active transport routes for drug delivery via conjunctiva

Carrier-mediated transport of drugs represents an area of growing interest to pharmaceutical scientists. Many carrier-mediated transport systems (mostly characterized in the small intestines) have been reported for absorption of amino acids, dipeptides, monosaccharides, monocarboxylic acids, phosphates, bile acids, and several water-soluble vitamins. These carrier-mediated transport systems play an important role in absorbing nutrients as well as their mimetic drugs [91]. The carrier-mediated drug transport system(s) endowed in conjunctival epithelium offer great advantages over passive diffusion, as conjunctiva is a tighter barrier than the intestinal epithelium. It is likely that the paracellular transport across conjunctiva may be more restricted, compared to that occurring in the small intestines due in part to more tighter paracellular routes. Na^+-coupled co-transport of D-glucose, L-Arg, nucleosides and monocarboxylate as well as H^+-coupled dipeptide co-transport processes are all reported to be localized in the mucosal aspect of the conjunctival epithelium. Utilizing these transporters for absorption of drugs and/or prodrugs as structure-mimetic substrates may become a feasible approach in ocular drug delivery in the coming years.

As L-Arg transport is inhibited by nitric oxide synthase (NOS) inhibitors (e.g. N^G-nitro-L-arginine methyl ester, N^G-monomethyl-L-arginine, and N^G-nitro-L-arginine (L-NA)) by $\sim$80% [32], L-NA appears to share the same transporter for L-Arg, i.e. the $B^{0,+}$ ($ATB^{0,+}$) system in the pigmented rabbit conjunctiva [92]. L-NA transport is saturable with a K_m of 0.35 mM and exhibits a 1:1 stoichiometry between Na^+ and L-NA. Moreover, the pattern of inhibition by other amino acids is the same as that afforded by L-Arg transport, i.e. basic and neutral amino acids can be used as inhibitors for such transport. As expected, L-Arg competitively inhibited L-NA transport with an apparent K_i of 0.034 mM, which compares well with the K_m of 0.07 mM for high affinity L-Arg transport found in conjunctiva [32]. As nitric oxide plays a cytostatic or cytotoxic role in the conjunctiva [93] and anterior uveal tract [94], it is attractive to attempt to deliver NOS inhibitors (e.g. L-NA) to conjunctiva proper and uveal tract via the conjunctival route to alleviate the NO burden. We envision that this approach is quite feasible, since $ATB^{0,+}$ is abundantly expressed in the mucosal aspect of conjunctiva [33]. System $B^{0,+}$ ($ATB^{0,+}$) recognizes almost all of naturally occurring neutral and cationic amino acids as substrates. Therefore, there is a wide range of choice for chemical modification of drugs, e.g. using prodrug approach to ensure optimal drug delivery. Hatanaka et al. [33] found that valacyclovir (L-valyl ester of acyclovir, which is used to treat cytomegalovirus (CMV) retinitis in AIDS patients) is transported as a substrate through the mouse $ATB^{0,+}$ in

human retinal pigment epithelial cells transfected with $ATB^{0,+}$ cDNA. Similarly, $ATB^{0,+}$ allows transport of acyclovir glutamic acid γ-ester (Acv-Glu) (which is modified at γ-carboxyl group of glutamate to exhibit a neutral charge profile). The IC_{50} values of valacyclovir and Acv-Glu for inhibition of glycine transport via $ATB^{0,+}$ are 0.7 and 3.3 mM, respectively. Therefore, valacyclovir has a higher affinity than that of Acv-Glu against system $ATB^{0,+}$ [33]. (It is worthwhile to note that valacyclovir is a better substrate for PepT1 than acyclovir and absorbed in small intestine via PepT1 quite well [95].) Although dipeptide transport process is present in the mucosal aspect of the conjunctiva, the expression level of dipeptide transporters in the conjunctiva seems to be rather low [48]. Stimulation schemes for such low levels of dipeptide transporters in the conjunctiva remain to be an obstacle.

As to nucleoside transport process(es), although CNT2 appears to be expressed in the mucosal aspect of the conjunctiva [35], targeting CNT2 as a drug delivery system for antiviral drugs may not be easily surmountable, as CNT2 exhibits a rather narrow specificity for substrates [36]. By contrast, targeting $ATB^{0,+}$ is likely to be a much more feasible strategy in the conjunctiva for ocular drug delivery using amino acid derivatives (including prodrugs).

4.1.3. Exploitation of endocytic transport pathway for ocular drug delivery via conjunctiva

The use of polymeric nanoparticles in the eye has gained considerable interest recently due to their stability, longer half-lives of elimination, and a wide range of application for the delivery of many drugs used to treat common ocular disorders. Nanoparticles have been utilized to enhance the absorption of therapeutic drugs, such as ibuprofen [96] and CSA [97], while reducing systemic side effect of cartrolol compared with commercial aqueous solution [98]. Qaddoumi et al. [99] found that poly (dl-lactide-*co*-glycolide, PLGA) nanoparticles are taken up by endocytosis in the apical side of RCEC. Although the endocytic mechanisms of nanoparticles in the conjunctiva have not been fully revealed yet, uptake of PLGA nanoparticles most likely occurs by adsorptive-type endocytosis [100]. In this regard, PLGA nanoparticles may indeed be a useful technique to apply ocular drug delivery via conjunctiva in the coming years.

4.2. Subconjunctival route

4.2.1. Subconjunctival ocular drug delivery

Subconjunctival ocular drug delivery represents another attempt to elevate intraocular drug concentrations and minimize the frequency of dosing. Compared with direct intravitreal injection, this approach is less risky to the patient and less invasive. Since sclera is much more permeable than conjunctiva [101,102], the formidable permeability barrier consisted of both cornea and conjunctiva can be avoided altogether in such approaches. Kompella et al. and Edelhauser and his group have been conducted with subconjunctival implants with nano-/microparticles and matrix materials [103–105].

The advantage of subconjunctival implants as opposed to subconjunctival injection of solution is the achievement of higher drug concentrations and sustained release of the drug into both vitreous humor and retinal areas. For example, dl-polylactide (PLA) nano- and microparticles containing budesonide (which inhibits the expression of vascular endothelial growth factor (VEGF) for treatment of angiogenesis in the retina) are reported to afford a sustained release of budesonide in vitro. Subconjunctival injection of PLA microparticles (3.6 μm) led to a much higher budesonide concentration in retina and vitreous humor over 14 days, compared with the solution form of dosing and PLA nanoparticle (345 nm) administration [103]. Edelhauser and his group [104,105] demonstrated that collagen matrix and fibrin sealant, compared to a simple drug solution form of dosing, provided a better controlled release of cisplatin and carboplatin, respectively, attaining higher drug concentrations after subconjunctival administration of rabbits in several ocular tissues including retina.

4.2.2. Transscleral/conjunctival iontophoresis

Iontophoresis is a technique of introducing drugs in the form of ionized substances into tissues non-invasively, by imposition of electric currents. Applying electric currents enhances drug permeability across the barrier [106]. Two types of iontophoresis have been utilized in ocular drug delivery: transcorneal and transscleral/conjunctival iontophoresis. Transcorneal iontophoresis has an advantage to enhance aqueous humor concentrations of drugs (including gentamicin, tobramycin, and vidarabine monophosphate), but limitation remains for delivering drugs in high enough concentrations to the posterior segment of the eye due to the presence of an iris-lens barrier to drug diffusion between the anterior and posterior chambers [107–109]. Transscleral/conjunctival iontophoresis affords higher concentrations of the delivered drugs in vitreous humor, dependent on applied current density and duration. Barza et al. [110] demonstrated that iontophoresis approaches enhance concentrations of ticarcillin, cefazolin, and gentamicin in vitreous humor. Applying 2 mA for 10 min, vitreal concentrations of cefazolin, ticarcillin, and gentamicin at 94–207 μg/ml in the normal rabbit eye are reported. Drug penetration shows a correlation with the strength of currents utilized (0.1–2 mA) and also with the duration (1–10 min) of iontophoresis procedure, but not with the concentration of the drug in solution [110]. Recently, this technique is applied to intraocular gene delivery as well [111,112]. Using transcorneoscleral iontophoresis approaches, fluorescein-labeled anti-NOSII oligonucleotides (ODNs) was detected in retina and NOSII was down-regulated following iontophoretic delivery of anti-NOSII ODNs in the rat model of endotoxin-induced uveitis, suggesting that iontophoresis facilitates the penetration of intact ODNs into the intraocular tissues

[112]. A companion approach is electroporation, a technique to permeabilize the cell membrane reversibly by using short duration-type electric-pulse for introduction of drugs, genes or monoclonal antibodies into the cytosol of cells. This technique was found to improve the efficacy of bleomycin, an antiproliferative drug, in reducing the intraocular pressure in glaucoma-filtering surgery [113, 114]. Safety issues and efficacy factors involved in such electrical approaches remain to be standardized for possible application of these techniques in vivo.

5. Conclusions

The development of formulations to deliver drugs topically to the eye must recognize the fact that these formulations will be in contact with both the cornea and conjunctiva. Up until 15 years ago, the influence of the conjunctiva in the overall disposition of the topically applied drugs was underestimated. This scenario began to shift when it was discovered that, possibly by virtue of their molecular properties that favor conjunctival access, a few compounds managed to trigger a pharmacological response in the posterior segment tissues when applied topically. We now know that the conjunctiva has properties of a moderately tight epithelium and exhibits both active net Cl^- secretion and Na^+ absorption. From the drug transport perspective, the conjunctiva is a tissue with a remarkable array of endogenous transport machinery to sample a wide range of molecules, including nanoparticles. Active Cl^- secretion into tear film is regulated by a number of factors including cAMP, Ca^{2+}, PKC, and nucleotides (via $P2Y_2$ type purinergic receptor). The cAMP-independent Cl^- secretion via stimulation of $P2(Y_2)$ receptors by (exogenous) UTP and UTP analogs may become quite useful therapeutically to induce alternative net Cl^- secretion (and thus concomitant fluid secretion) in dry eye or cystic fibrosis. Na^+-coupled transport processes for absorption of D-glucose, amino acids, nucleosides and monocarboxylates, and H^+-coupled dipeptide transport process are all present in the mucosal aspect of conjunctiva, suggesting their important roles in transconjunctival homeostatic balance in ions and water of tear fluid as well as volume regulations of the involved cells.

System $B^{0,+}$ ($ATB^{0,+}$) recognizes almost all amino acids (sans anionic amino acids) as a substrate and is expressed abundantly in the conjunctiva, making it very attractive to target $ATB^{0,+}$ for drug absorption via transconjunctival route. This may become quite a feasible strategy using amino acid derivatives including prodrugs for ocular delivery. Subconjunctival administration of nano-/microparticles and matrix materials offer the opportunity to achieve sustained drug concentration in the vitreous humor and retina for a prolonged time period, perhaps, up to several weeks. Transscleral/conjunctival iontophoresis is a new technique for the enhancement of drug absorption into the vitreous humor and may be utilized for enhancing gene expression in various regions of the eye that may not be easily reachable by conventional means. As more data become available for conjunctival transport (patho)physiology, as compared to the well known corneal arena, we may be able to utilize more easily and effectively the conjunctival routes for drug delivery into the posterior portion of the eye.

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